

ON THE CHEMICAL COMPOSITION OF THE BROWN ALGA *HIMANTHALIA ELONGATA* (L.) S. F. GRAY

RAYMOND F. JONES

Botany Department, King's College, Newcastle-upon-Tyne, England

In recent years the commercial importance of many seaweeds has resulted in considerable work being carried out on the chemical composition of marine algae (Black, 1953). *Himanthalia elongata*, although a common dioecious fucoid along the coasts of northern England, does not occur in sufficient quantities to render it of commercial importance. As a result it has been little investigated. Colin and Ricard (1930) first recorded the chemical composition of *Himanthalia*, but their work is of little value since no indication of the size or condition of the plants analyzed was given. Moss (1952), however, noted that during the spring a variation in chemical composition occurred with the stage of development of *Himanthalia* collected at the same time from the same habitat. No distinction of sex was made in the samples, although for *Fucus vesiculosus* Moss (1950) found that the male receptacles were higher in total nitrogen than the female receptacles.

The present work is concerned with the chemical constituents of both male and female plants of *Himanthalia* and with the variation of such constituents along the length of the plant.

MATERIALS AND METHODS

Samples of *H. elongata* were collected from St. Mary's Island, Northumberland at low water as the tide receded. All samples were returned to the laboratory in sea water thus ensuring that the plants, prior to analysis, were fully turgid. For analysis samples were sorted into developmental and sexual stages. Each sample contained at least 100 individuals. Fresh weight determinations were made after removing the adhering moisture from the surface of the plants with cheese cloth. The plant material was loosely packed in shallow aluminum trays, placed in an electric oven through which a current of air was forced. The material was dried for one hour at 90° C. (to inactivate enzymes) and then for a further 23 hours at a temperature range of between 60 and 65° C. On cooling to room temperature the dried material was ground in a Christie & Norris No. 8 Laboratory Mill, fitted with a 1/64-inch perforated screen, giving a powder which practically all passed 90 mesh. Dry weight determinations were made on 0.1–0.2 gm. samples of the above dried milled weed.

Total nitrogen estimations were obtained by the micro-Kjeldahl method of Kelley, Hunter and Sterges (1946) using a Markham Still for distillation. The soluble non-protein nitrogen was extracted from the dried material with water for 6 hours at laboratory temperature with constant shaking, then stored at 5° C. for 18 hours with occasional shaking. After filtering, the proteins were precipitated with sufficient trichloroacetic acid to produce a 2.5% solution. The extract was

filtered through Whatman 42 filter paper and made up to 250 ml. This method of extraction gave consistent results for total non-protein nitrogen. The soluble non-protein nitrogen was determined using the method of Langer (1952). Of the soluble non-protein constituents, volatile base and amide nitrogen values were obtained by the method of Channing and Young (1953). The free α -amino nitrogen was estimated using the technique employed by Sobel, Hirschman and Besman (1945). Peptide nitrogen was determined using the method of Haas, Hill and Russell-Wells (1938); Sideris and Young (1946).

The carbohydrate and related constituents were determined by the methods of Cameron, Ross and Percival (1948).

Inorganic ash content of the dried material was estimated after preliminary sulphuric acid treatment as described by Piper (1950).

Free amino acids were extracted from the dried milled weed (one gm.) by shaking three times with 75% ethanol (100 ml.). The solution was centrifuged after each extraction and the combined extracts passed through Zeo-Karb 225 and the free amino acids and peptides eluted with ammonium hydroxide (1 N). The eluates were dried *in vacuo* over calcium chloride and taken up in HCl (0.1 N) and chromatographed. Combined amino acids in the dried material (0.5 gm.) were hydrolyzed in a sealed tube with HCl (6 N; 5 ml.) for 48 hours at 105° C. Excess acid was removed by drying *in vacuo* over calcium chloride and sodium hydroxide. Samples were ion exchanged as above and similarly chromatographed. For the detection of tryptophane, alkaline hydrolysis with barium hydroxide (saturated at room temperature) was used.

Free sugars were extracted from the dried material (3 gm.) after refluxing with 75% ethanol (100 ml.) for 6 hours. After filtration the extract was passed through the ion exchange resins Zeo-Karb 225 and De-Acidite E, reduced in volume and aliquots used for paper chromatography. Sugars on hydrolysis were determined by hydrolyzing the dried milled weed (one gm.) with HCl (50 ml.; 2 N) for 8 hours using a reflux condenser. Following filtration the extract was subjected to ion exchange resins as for free sugars and chromatographed.

Extraction of the organic acids was accomplished by shaking the dried milled weed (3 gm.) with water (100 ml.) for 6 hours. The mixture was filtered and 80% ethanol added to precipitate protein. The precipitate was removed by centrifugation and the supernatant passed through Zeo-Karb 225 followed by De-Acidite E. The acidic compounds were eluted from the latter resin with ammonium hydroxide (1 N).

Methods in paper chromatography were those described by Block (1952) and Ranson (1955).

RESULTS

1. *Qualitative determination of some chemical constituents*

a) Amino acids, free and combined

The presence of only a few free amino acids, namely alanine, aspartic and glutamic acids, was detected on one and two dimensional chromatograms. The normal plant amides asparagine and glutamine were not detected. On hydrolysis with HCl (6 N) for 48 hours the alcoholic extracts gave compact spots corresponding

to aspartic, glutamic, alanine and traces of glycine, serine and the leucines, suggesting the presence of these acids in peptide formation. The intensity of the glutamic and alanine spots was greatly increased after hydrolysis, suggesting that they occur in peptides as well as in the free state. Other alcoholic extractions of different developmental stages of *Himantalia* gave similar results.

A chromatographic analysis of the peptide fraction precipitated from the water-soluble non-protein extracts with mercuric acetate and treatment with H_2S , as employed by Haas, Hill and Russell-Wells (1938), after hydrolysis revealed the presence of the following amino acids: glutamic (strong), alanine (strong), aspartic (weak-strong), serine (weak), glycine (weak), leucine (trace), isoleucine (trace).

In acid and alkaline hydrolysates of the dried milled weed from all stages of development the following amino acids were detected: alanine, arginine, aspartic, cysteic, glycine, glutamic, histidine, iso-leucine, leucine, lysine, methionine, methionine sulphoxide, phenylalanine, proline, serine, threonine, tryptophane, tyrosine and valine. In the majority of samples analyzed histidine was present only in traces, its presence being confirmed with the Pauly diazotized sulphanilic reagent (Consden *et al.*, 1946). The isatin dip method of Jepson and Smith (1953) confirmed the presence of proline and the absence of hydroxyproline. The copper carbonate technique for the detection of non α -amino acids (Crumpler and Dent, 1949) failed to show the presence of either β -alanine or γ -amino butyric acid in the free state or in combination.

b) Carbohydrates

Very little sugar could be detected in the free state. Spots with R_F values similar to fucose and galactose were obtained. Glucose, fucose, arabinose, xylose, and galactose were found to be present on chromatograms of the acid-hydrolyzed weed samples. The sugar alcohol mannitol was detected on both sugar chromatograms.

c) Organic acids

Very few organic acids were detected in extracts of *Himantalia*. Citric acid was the chief organic acid detected. In some cases traces of malic acid were found to be present. No other acids of the Krebs cycle were detected. On all chromatograms a large acidic spot was found to exist in appreciable quantity. In tertiary amyl-formic acid-water solvent, the $R_F = 0.20$, in the propanol- NH_3 solvent, the $R_F = 0.51$. With silver nitrate reagent it gave an intense white color on a brown background. It was negative to ninhydrin and Hane's phosphate reagent. On the chromatograms it ran in close association with standard gluconic acid. The large quantities of alginic acid present in the weed samples leads one to think that it may be mannuronic acid, which would be the precursor of the larger alginic acid molecule. Unfortunately a standard sample of mannuronic acid could not be obtained. Another acidic spot was found to be orthophosphate.

2. Variation in nitrogenous constituents

During July, 1953, samples of *Himantalia* were collected from St. Mary's Island. After preliminary sorting, drying and grinding these plants were analyzed

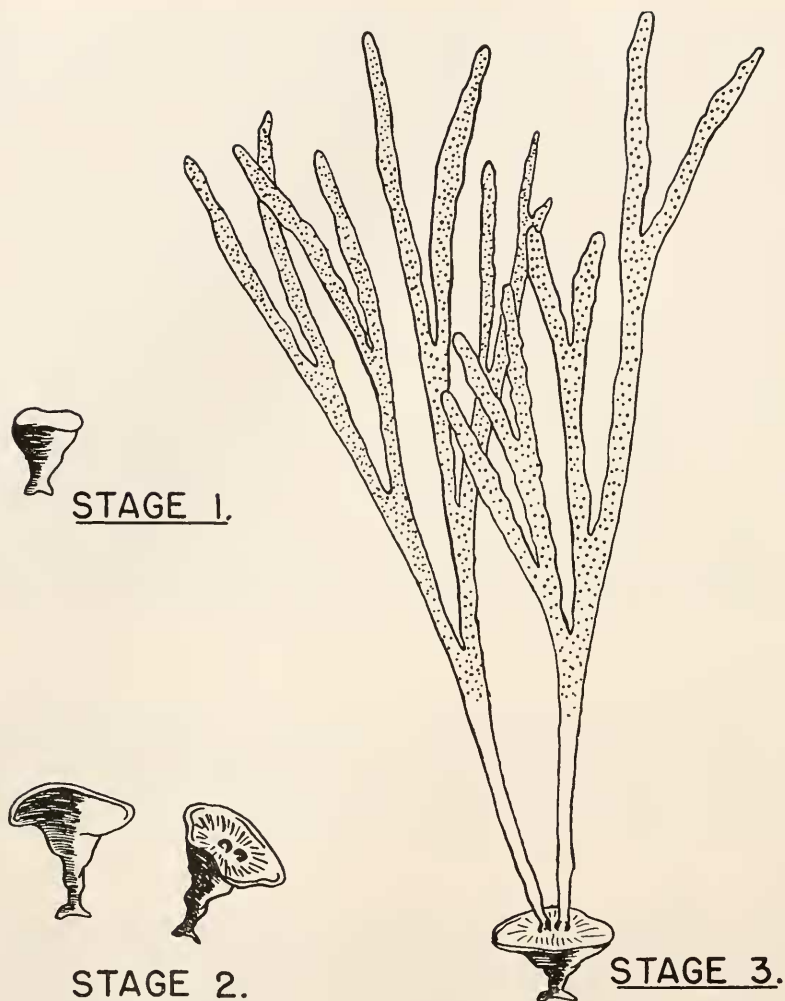


FIGURE 1. Stages of development of *Himanthalia* used in the chemical analysis, July, 1953: Stage 1. Young vegetative buttons, 1–1.5 cm. in length and tubular in form. Stage 2. Fully grown vegetative buttons. Stage 3. Mature plants composed of buttons, from which were given off long mature receptacles some 50–60 cm. in length. The receptacles were separated from the buttons, and further sorted into male and female for separate analysis.

for total nitrogen, protein nitrogen and non-protein nitrogen. The stages of development used in this analysis are shown in Figure 1.

The results (Table I) are interesting insofar as they show that the male receptacles contain higher nitrogen values than the female receptacles. The higher nitrogen content of the male is associated with the protein fraction. The vegetative plants, *i.e.*, the buttons, contain a higher percentage of soluble non-protein nitrogen than the mature buttons in stage 3. This appears to consist mainly of peptide and free amino acid nitrogen.

TABLE I

Variation of nitrogen in plants from St. Mary's Island, July, 1953

i) Total nitrogen, protein and non-protein nitrogen

| Stage of development | Total N | Protein N | Non-protein N | Non-protein N as % total N |
|----------------------|---------|-----------|---------------|----------------------------|
| 1. Buttons | 6.75 | 5.84 | 0.91 | 13.5 |
| 2. Buttons | 12.30 | 9.85 | 2.45 | 19.9 |
| 3. Buttons | 12.15 | 10.92 | 1.23 | 10.1 |
| Female receptacles | 13.45 | 10.54 | 2.91 | 21.6 |
| Male receptacles | 15.00 | 12.12 | 2.89 | 19.3 |

ii) Soluble non-protein constituents

| Stage of development | Volatile base N | Peptide N | α -Amino N |
|----------------------|-----------------|-----------|-------------------|
| 1. Buttons | 0.20 | 0.46 | 0.43 |
| 2. Buttons | 0.62 | 1.39 | 0.59 |
| 3. Buttons | 0.31 | 0.97 | 0.32 |
| Female receptacles | 0.63 | 1.54 | 0.93 |
| Male receptacles | 0.60 | 1.40 | 0.69 |

3. Further analysis of male and female receptacles

During September, 1954, when plants were ripe, male and female individuals were collected and the receptacles analyzed separately. The results are given in Table II.

In order to obtain antherozooids and oogonia for analysis, male and female receptacles were separately washed with filtered sea water and allowed to dry out for several hours. During this process gelatinous masses of gametes appeared at the ostioles of the conceptacles. The plants were then placed in separate Pyrex glass containers with filtered sea water and the plants allowed to extrude their gametes. The gametes were separated from the sea water by centrifugation, dried at 90° C.

TABLE II

Analysis of male and female receptacles, Sept., 1954

| | Male receptacles | Female receptacles |
|------------------|-------------------------|-------------------------|
| Mean height | 84.7 cm. per unit plt. | 86.5 cm. per unit plt. |
| Mean width | 0.8 cm. per unit plt. | 0.8 cm. per unit plt. |
| Fresh weight | 122.3 gm. per unit plt. | 119.0 gm. per unit plt. |
| Dry weight | 15.6 gm. per unit plt. | 15.2 gm. per unit plt. |
| Total nitrogen | 19.7 | 16.6 |
| Protein nitrogen | 14.8 | 12.0 |
| Non-protein N | 4.9 | 4.6 |
| Mannitol | 65.1 | 66.8 |
| Alginic acid | 132.4 | 178.9 |

} mg. per gm.
dry weight

} mg. per gm.
dry weight

TABLE III

Analysis of male and female gametes, Sept., 1954

| Constituent | Male gametes (mg./gm. dry wt.) | Female gametes (mg./gm. dry wt.) |
|----------------------|-----------------------------------|-------------------------------------|
| Total nitrogen | 69.33 | 25.97 |
| Protein nitrogen | 55.81 | 13.83 |
| Non-protein nitrogen | 15.52 | 12.14 |
| Total ash | 826.60 | 819.60 |

for one hour, followed by 23 hours at 60° C. An analysis of the nitrogenous constituents and total inorganic ash is given in Table III.

The results of these two analyses show that between the male and female receptacles there is little difference in size and weight. The protein nitrogen is higher in the male than in the female, thus confirming the results found for the receptacles collected in July, 1953. The male gametes are appreciably higher in protein content than the female gametes, which suggests that the high protein content of the male receptacles is in all probability due to the high protein content of the gametes. The exceptionally high ash content of the gametes is of great interest. The alginic acid is higher in the female receptacles than in the male.

4. *Variation of chemical constituents along the length of the plant*

One hundred plants were collected from St. Mary's Island during April, 1954, and divided into three regions for analysis of the various chemical constituents. The divisions were as follows:

Region A. Mature buttons of the plant.

Region B. Sterile base of receptacle, the length of which was approximately 5 cm. from the surface of the button.

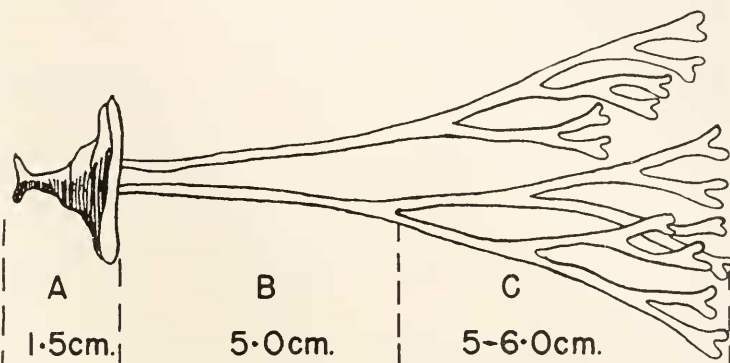
Region C. Remainder of the receptacle. This region contained developing conceptacles which are differentiated behind the growing apices. Length of region varied from 5.0 to 6.0 cm.

The results in Figure 2 indicate that variations occur along the length of the plant. Of the nitrogenous constituents analyzed, the young developing receptacle tips and conceptacles of region C possess higher values than the base of the receptacle (region B) which is sterile. The vegetative button (region A) possesses considerably lower amounts of nitrogen than those recorded for either region of the receptacle. The tips and base of the receptacle are higher in mannitol than the vegetative button. The button, however, is higher in alginic acid and laminarin content. The sterile base of the receptacle contains more alginic acid than the tips of the receptacles.

GENERAL DISCUSSION

The qualitative determination of some of the chemical constituents recorded for *Himanthalia* is in accordance with the findings of other workers. The detection of only a few amino acids in the free state is to be found in other marine algae (Channing and Young, 1953; Coulson, 1953a; Smith and Gordon Young, 1953). The

presence of peptides, particularly of glutamic acid and alanine, affords further evidence that marine algae differ from fresh water algae and land plants in that they show a preference for peptides in place of amino acids (Dent, Stepka and Steward, 1947; Steward and Thompson, 1950; Fowden, 1951). The typical land plant



| CONSTITUENT | BUTTON | BASE of RECEPTACLE | UPPER RECEPTACLE |
|---------------|--------|--------------------|------------------|
| TOTAL N | 13.51 | 20.25 | 28.70 |
| PROTEIN N | 11.87 | 15.56 | 20.29 |
| NON PROTEIN N | 1.64 | 4.79 | 8.41 |
| VOL. BASE N | 0.39 | 0.66 | 0.77 |
| FREE AMINO N | 0.50 | 1.20 | 1.19 |
| PEPTIDE N | 0.71 | 1.59 | 2.12 |
| ALGINIC ACID | 215.40 | 166.80 | 119.00 |
| MANNITOL | 60.77 | 74.38 | 79.62 |
| LAMINARIN | 21.18 | 8.15 | 9.61 |

(mg. per gm. dry wt.)

FIGURE 2. Variation of constituents along length of plant, April, 1954.

amides asparagine and glutamine were not detected in *Himanthalia*. The amino acids present in the hydrolysates of the marine alga were similar to those found by other workers for the Phaeophyceae (Channing and Young, 1953; Coulson, 1953b; Smith and Gordon Young, 1953), and for fresh water algae (Fowden, 1954). They are similar to those in land plants. The sugars fucose and galactose, and the sugar-alcohol mannitol were detected in the free state. The recent work of Lindberg (1953) and Lindberg and Paju (1954) has shown the presence of mannitol and mannitol glucosides to occur in other fucoids. It is realized, however, that the presence of fucose and galactose may be the result of polysaccharide hydrolysis during the drying process. The sugars glucose, fucose, arabinose, xylose and galactose present in the acid hydrolysates are in keeping with the results of other workers (Connell, Hirst and Percival, 1950; Percival and Ross, 1950; Ross, 1953; Dewar, 1954), in that they are the component sugars of laminarin, fucoidin and cellulose. Little work has been published on the organic acids of marine algae apart from the work of Créach (1952), who detected citric acid in a number of fucoids. The presence of citric acid and traces of malic acid found in *Himanthalia* are commonly found in land plants. The presence of free orthophosphate has been noted for other algae (Blinks, 1951).

The male receptacles of *F. vesiculosus* were shown by Moss (1950) to be higher in nitrogen content than the female receptacles. For *Himanthalia* similar results have been obtained, the male receptacles containing more nitrogen, particularly protein nitrogen, than did the female receptacles. The male gametes of *Himanthalia* were also found to contain more nitrogen than female gametes. This is similar to results published by Sosa-Bourdouil (1940), who, for *F. vesiculosus*, found the male gametes to contain twice as much nitrogen as the female gametes on a dry weight basis. The high concentration of nitrogen in the male gametes seems therefore to be the factor contributing to the high nitrogen content of the male receptacles.

The analysis of the chemical constituents along the length of the plant showed the tissues of mature buttons to be lower in nitrogen and mannitol but higher in alginic acid and laminarin than were the receptacles on the same plant. The apical regions of the receptacles were high in mannitol and nitrogen content, particularly protein. The basal sterile region of the receptacle was similarly high in mannitol, but lower in nitrogen than the uppermost region. The higher soluble non-protein nitrogen content of the upper receptacle region is, no doubt, correlated with the synthesis of protein for growth and gamete formation. The results may be compared with those of Moss (1950), who for *F. vesiculosus* found that the young receptacles were higher in nitrogen content than the vegetative regions of the thallus, which is in contrast with *Himanthalia* for in this species the fertile tissue was higher in nitrogen content than the vegetative button. Jacobi (1954) has recorded high nitrogen values for the apical regions of *F. vesiculosus* throughout the year. For *Laminaria saccharina*, the same author found a higher nitrogen content in the meristematic region of the thallus than in the more mature tissues of the frond. The findings of Moss (1950) and Jacobi (1954), together with the present author's results for *Himanthalia*, indicate that actively growing regions and regions of reproductive development are higher in nitrogen content than the more mature vegetative tissues of the plant.

The work in this paper forms part of the programme of research and development on marine algae by the Institute of Seaweed Research and the author is indebted to the Institute for permission to publish. The author also wishes to thank Dr. Betty L. Moss, under whose supervision this work was carried out.

SUMMARY

1. The qualitative determination of amino acids, sugars and organic acids in *Himantalia elongata* has been carried out.
2. Mature vegetative tissues were found to be lower in nitrogen content than actively growing reproductive tissues.
3. The high nitrogen content of the male receptacles was correlated with the higher protein nitrogen content of the male gametes.
4. Vertical gradients of organic constituents were found to occur along the length of the individual plant. The mature button was lower in nitrogen and mannitol but higher in alginic acid and laminarin than the receptacles of the same plant. The apical regions of the receptacles were high in mannitol and nitrogen content, particularly protein. The basal sterile region of the receptacle was similarly high in mannitol, but was lower in nitrogen than the uppermost region.

LITERATURE CITED

- BLACK, W. A. P., 1953. Constituents of marine algae. *Ann. Reports Chem. Soc.*, **50**: 322-355.
- BLINKS, L. R., 1951. Physiology and biochemistry of algae. Manual of Phycology, Chron. Bot. Co.
- BLOCK, R. J., 1952. Paper chromatography. Academic Press Inc., New York.
- CAMERON, M. C., A. G. ROSS AND E. G. V. PERCIVAL, 1948. Methods for the routine estimation of mannitol, alginic acid, laminarin and combined fucose in seaweeds. *J. Soc. Chem. Ind.*, **67**: 161-164.
- CHANNING, D. M., AND G. T. YOUNG, 1953. Amino acids and peptides. Part X. The nitrogenous constituents of some marine algae. *J. Chem. Soc.*, pp. 2481-2491.
- COLIN, P., AND R. RICARD, 1930. Glucides et dérivés glucidiques des algues brunes. *C. R. Acad. Sci. Paris*, **190**: 1514-1516.
- CONNELL, J. J., E. L. HIRST AND E. G. V. PERCIVAL, 1950. The isolation of laminarin. Part I. An investigation on laminarin isolated from *Laminaria cloustoni*. *J. Chem. Soc.*, pp. 3494-3500.
- CONSDEN, R., A. H. GORDON AND A. J. P. MARTIN, 1946. Ionophoresis in silica jelly. *Biochem. J.*, **40**: 33-44.
- COULSON, C. B., 1953a. Amino acids of marine algae. *Chem. and Ind.*, pp. 971-972.
- COULSON, C. B., 1953b. Proteins of marine algae. *Chem. and Ind.*, pp. 997-998.
- CRÉACH, P. V., 1952. On the presence of citric acid in seaweeds. *1st. Int. Seaweed Symp. (Edin.)* pp. 42-43.
- CRUMPLER, H. R., AND C. E. DENT, 1949. Distinctive test for non α -amino acids in paper chromatography. *Nature*, **164**: 441-442.
- DENT, C. E., W. STEPKA AND F. C. STEWARD, 1947. Detection of the free amino acids of plant cells by partition chromatography. *Nature*, **160**: 682-683.
- DEWAR, E. T., 1954. The occurrence of D-xylose and D-galactose in brown seaweeds. *Chem. and Ind.*, pp. 785-786.
- FOWDEN, L., 1951. Amino acids of certain algae. *Nature*, **167**: 1030-1031.
- FOWDEN, L., 1954. A comparison of the compositions of some algal proteins. *Ann. Bot., N.S.*, **18**: 257-266.
- HAAS, P., T. G. HILL AND B. RUSSELL-WELLS, 1938. On certain simple peptides occurring in marine algae. *Biochem. J.*, **32**: 2129-2133.

- JACOBI, G., 1954. Die Verteilung des Stickstoffs in *Fucus vesiculosus* und *Laminaria saccharina* und deren Abhängigkeit vom Jahresrhythmus. *Kieker Meeresforschungen*, Band X, Heft 1: 37-57.
- JEPSON, J. B., AND I. SMITH, 1953. Multiple dipping procedures in paper chromatography: a specific test for hydroxyproline. *Nature*, **172**: 1100-1101.
- KELLEY, O. J., A. S. HUNTER AND A. J. STERGES, 1946. Determination of nitrogen, phosphorus, potassium, calcium and magnesium in plant tissues. Semi-micro wet digestion method for large number of samples. *Ind. and Eng. Chem. (Anal. Ed.)*, **18**: 319-322.
- LANGER, R. H. M., 1952. A study of nitrogenous metabolites in tillering plants of *Triticum vulgare*. Ph.D. Thesis (Univ. of Durham, England).
- LINDBERG, B., 1953. Three new carbohydrates isolated from *Fucus vesiculosus*. *Acta Chem. Scand.*, **7**: 1119-1122.
- LINDBERG, B., AND J. PAJU, 1954. Low-molecular carbohydrates in algae. IV. Investigation of *Pelvetia canaliculata*. *Acta Chem. Scand.*, **8**: 817-820.
- MOSS, B. L., 1950. Studies in the genus *Fucus*. II. The anatomical structure and chemical composition of receptacles of *Fucus vesiculosus* from three contrasting habitats. *Ann. Bot., N.S.*, **14**: 396-410.
- MOSS, B. L., 1952. Variations in chemical composition during the development of *Himanthalia clongata* (L.) S. F. Gray. *J. Mar. Biol. Assoc. U.K.*, **31**: 29-34.
- PERCIVAL, E. G. V., AND A. G. ROSS, 1950. Fucoidin. Part I. The isolation and purification of fucoidin from brown seaweeds. *J. Chem. Soc.*, pp. 717-720.
- PIPER, C. S., 1950. Soil and plant analysis. Monograph Univ. of Adelaide.
- RANSON, S. L., 1955. Non-volatile mono-, di- and tricarboxylic acids. *Modern Methods of Plant Analysis*, Vol. 2, pp. 539-584.
- ROSS, A. G., 1953. Some typical analyses of red seaweeds. *J. Sci. Food Agric.*, **7**: 333-335.
- SIDERIS, C. P., AND H. Y. YOUNG, 1946. Effects of iron on certain nitrogenous fractions of *Ananas cosmus*. *Plant Phys.*, **21**: 75-94.
- SMITH, D. G., AND E. GORDON YOUNG, 1953. On the nitrogenous constituents of *Fucus vesiculosus*. *J. Biol. Chem.*, **205**: 849-858.
- SOBEL, A. E., A. HIRSCHMAN AND L. BESMAN, 1945. A convenient micro-titration method for the estimation of amino acids. *J. Biol. Chem.*, **161**: 99-103.
- SOSA-BOURDOUIL, A. AND D., 1940. Sur les *Fucus* et la composition de leurs fructifications. *Bull. Lab. Mar. Dinard*, **23**: 43-47.
- STEWART, F. C., AND J. F. THOMPSON, 1950. The nitrogenous constituents of plants with special reference to chromatographic methods. *Ann. Rev. Plant Phys.*, **1**: 233-264.